

## Endonuclease-independent DNA Mismatch Repair Processes on the Lagging Strand

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### **Abstract:**

DNA mismatch repair (MMR) pathways coordinate the excision and re-synthesis of newly-replicated DNA if a mismatched base-pair has been identified by protein MutS or MutS homologues (MSHs) after replication. DNA excision during MMR is initiated at single-strand breaks (SSBs) *in vitro*, and several redundant processes have been observed in reconstituted systems which either require a pre-formed SSB in the DNA or require a mismatch-activated nicking endonuclease to introduce a SSB in order to initiate MMR. However, the conditions under which each of these processes may actually occur in living cells have remained obscured by the limitations of current MMR assays. Here we use a novel assay involving chemically-modified oligonucleotide probes to insert targeted DNA ‘mismatches’ directly into the genome of living bacteria to interrogate their replication-coupled repair processes quantitatively in a strand-, orientation-, and mismatched nucleotide-specific manner. This ‘semi-protected oligonucleotide recombination’ (SPORE) assay reveals direct evidence in *Escherichia coli* of an efficient endonuclease-independent MMR process on the lagging strand—a mechanism that has long-since been considered for lagging-strand repair but never directly shown until now. We find endonuclease-independent MMR is coordinated asymmetrically with respect to the replicating DNA—directed primarily from 3'- of the mismatch—and that repair coordinated from 3'- of the mismatch is in fact the primary mechanism of lagging-strand MMR. While further work is required to explore and identify the molecular requirements for this alternative endonuclease-independent MMR pathway, these findings made possible using the SPORE assay are the first direct report of this long-suspected mechanism *in vivo*.

**Keywords:** DNA mismatch repair | Oligonucleotide recombination | Replication | Methyl-directed mismatch repair | *Escherichia coli*

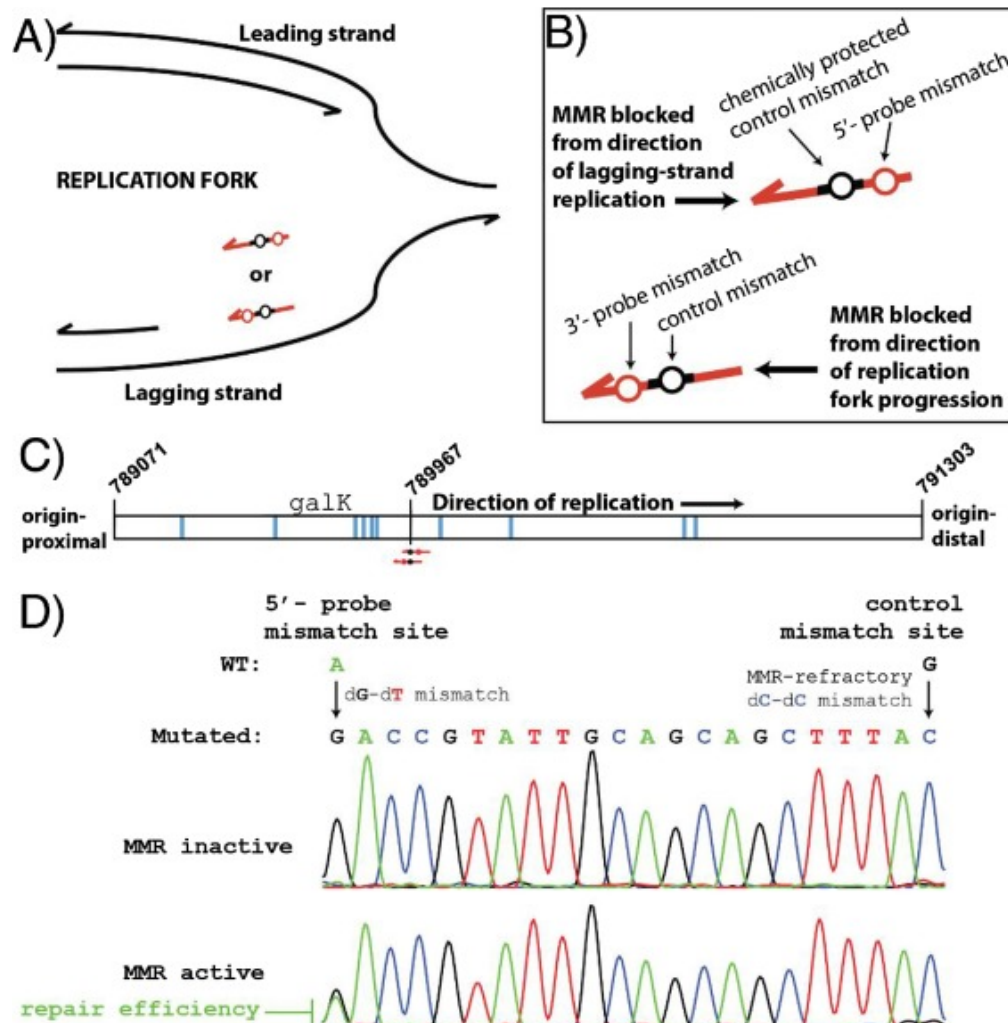
### **Article:**

#### **1. Introduction**

In a mechanism that is highly conserved from bacteria to humans, the DNA mismatch repair (MMR) pathway coordinates the excision and correct re-synthesis of tracts of DNA that contain mismatched nucleotides or insertion/deletion (indel) loops that may have been erroneously generated during replication [1], [2]. At the initiation of the conserved mechanism, protein MutS or MutS homologues (MSH) bind to the replication errors, after which a latent nicking endonuclease (MutL $\alpha$  in humans and MutH in *Escherichia coli*, *e.g.*) is authorized to introduce single-strand breaks (SSBs) on the newly-replicated strand [3], [4], [5], [6], [7]. These breaks can serve as entry points for DNA helicases and exonucleases to begin excision of the DNA from the SSB through the error in a process termed “long-patch repair” (LPR), since long contiguous stretches of DNA (hundreds of nucleotides) can be digested and resynthesized between the two sites.

While disruption of MutS/MSH or nicking endonuclease activity results in strong ‘mutator’ phenotypes *in vivo* [4], [8], [9], [10], nicking endonuclease activity is not necessary to initiate MMR *in vitro* if the mismatched DNA already contains a SSB [3], [5], [11], [12]. Because breaks occur naturally between Okazaki fragments on the lagging strand as it is synthesized discontinuously, it has been proposed that MMR complexes may coordinate MMR on the lagging strand at those breaks in the absence of MMR endonucleases [13], [14], [15]. In particular, there are several lines of evidence of an endonuclease-independent mechanism MMR from studies in eukaryotes (reviewed in [13]) to suggest that the 5'- end of the Okazaki fragment may be used to coordinate MMR during the processing of its 5'- RNA primer and Okazaki maturation, but this mechanism has never been directly demonstrated to occur in living cells.

To investigate the possibility of an endonuclease-independent repair on the lagging strand *in vivo*, we require a newly-developed assay, which we call a ‘semi-protected oligonucleotide recombination’ (SPORE) assay [16], that uniquely allows us to introduce targeted ‘replication errors’ directly into genomic DNA at the replication fork and to quantify their repair in a strand-, orientation-, and mismatched nucleotide-specific manner (Fig. 1). By performing this SPORE assay in *E. coli*—a model organism for MMR—and inserting a ‘mismatch’ onto the replicating lagging strand, here we are able to directly identify an efficient MMR-endonuclease-independent mismatch repair in *mutH*-knockout strains. Endonuclease-independent repair of lagging-strand mismatches is coordinated primarily from 3'- of the mismatch and weakly contributes to repair initiated from the 5'- end of the Okazaki fragment. Repair coordinated from the 5'- end of the error is strongly MMR endonuclease-dependent; however evidence from the SPORE assay suggests that LPR coordinated from the 3'- polarity—in the direction of lagging-strand replication (Fig. 1B) and which does not require MutH—represents the primary mechanism of MMR on the lagging strand. While further work is required to specifically identify the molecular requirements and epigenetic repair signals used to coordinate endonuclease-independent MMR, these findings provide the first direct report in living cells of what appears to be the primary mechanism for lagging-strand MMR.



**Figure 1.** A “semi-protected oligonucleotide recombination” (SPORE) assay to quantify mismatch repair (MMR) efficiency *in vivo* (see text for details). (A) Chemically modified oligonucleotides that are mostly complementary to the lagging strand template are transfected into *E. coli* and become incorporated into the lagging strand at the replication fork in a process that is proofread by the MMR pathway (Ref. [16] and references therein). (B) The SPORE oligonucleotide (oligo) contains a ‘probe’ mismatch that introduces a silent mutation in the *galK* gene if left unrepaired, as well as a ‘control’ mismatch that is weakly recognized and not repaired by *E. coli* MMR (a dC-dC mismatch [17]) but designed to introduce a selectable mutation, by correcting an amber mutation in the galactose kinase gene *galK* [18]. The control mismatch is flanked by multiple phosphorothioate bonds [19] (black) to protect against degradation during MMR and to block long-patch repair that initiates from the opposite side as the probe mismatch. (C) Genomic map of the local d(GATC) sites (blue lines) near the sites complementary to the SPORE oligos. (D) In the SPORE assay, successful transformants are screened for a MMR-refractory control mutation by enrichment in a galactose media. The directional repair efficiency is determined from the sequencing signal at the site of the ‘probe’ mismatch in a manner that robustly accounts for imperfect transformation efficiencies, non-specific SPORE oligo degradation, and chromatogram noise that may occur (see Supplementary Methods).

## 2. Material and methods

### 2.1. Materials

*E. coli* strains SIMD50 (W3110 *galK*<sub>tyr145UAG</sub>  $\Delta$ *lacU169* [ $\lambda$  cI857  $\Delta$ (*cro-bioA*) (*int-cIII*<>*bet*)]), SIMD90 (SIMD50 *mutS*<>*cat*), and HME61 (W3110 *galK*<sub>tyr145UAG</sub>  $\Delta$ *lacU169* [ $\lambda$  cI857  $\Delta$ (*cro-bioA*) (*mutH*<>*amp*)] were obtained as a generous gift of the laboratory of Don Court (National Cancer Institute, Frederick, MD). M9 minimal salts (5x) were obtained from Sigma-Aldrich Co. M63 media (3% KH<sub>2</sub>PO<sub>4</sub> w/w, 7% K<sub>2</sub>HPO<sub>4</sub> w/w, 2% (NH<sub>4</sub>)SO<sub>4</sub> w/w, 2% D-galactose w/w, 1 mM MgSO<sub>4</sub>, 0.5 mg/L FeSO<sub>4</sub>, 1 mg/L D-biotin) was prepared as previously described [20]. For galactose-selective M63 media, 2% D-galactose w/w was added, and for galactose-selective agar Plates 15 g of agar was added to 1 L of this solution. For 2-deoxygalactose (DOG) agar plates [21], we used 1 L M63 without galactose, 15 g agar, 2 mL glycerol (0.2%; Fischer), and 2 g DOG. Taq 2X MasterMix was obtained by New England Biolabs (Ipswich, MA) and used for all PCR reactions. Gene Pulser(R)/MicroPulser(tm) Electroporation Cuvettes, 0.1 cm gap were obtained from Bio-Rad Laboratories. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) without 5'- phosphorylation (as these ends are readily generated *in vivo* by nucleolytic trimming [22]) and with standard desalting, and they were used without further purification.

### 2.2. Generation of *E. coli* strain variants

Generation of strain SIMD50 MutSD835R was described previously [16]. Strains SIMD50 *galK* g.348G>A and SIMD50 *galK* g.158G>C 348G>A, with the first and first two origin-distal d(GATC) sites (Fig. 1C) removed by synonymous mutations, respectively, were generated by Red-mediated oligo-mediated recombination according to the standard protocol [23], [24] as previously described [16]. First SIMD50 was transformed *via* electroporation with a phosphorothioated version of oligo *I44* from [25] to correct the amber mutation in the *galK* gene (see Table S1, Supplementary Materials), and plated on galactose-selective agar plates immediately after 30 min outgrowth followed by a wash with M9 media. Colonies were picked and sequenced to confirm, then re-transformed *via* Red-mediated oligo-mediated recombination with oligos *galK* +85 (which introduces a stop codon into the *galK* gene near first origin-distal d(GATC) site) and *galK* +275 (introduces a synonymous mutation in the second origin-distal d(GATC) site), then plated immediately after 30 min outgrowth and washing onto DOG agar plates. Colonies were screened by PCR using primers 5'-AGCCACATATTGCCATC-3' and 5'-ATAACGCCACCGAATAC-3' and restriction fragment length analysis using restriction enzyme MboI (New England Biolabs). Based on the restriction pattern by MboI by agarose gel electrophoresis, we identified colonies with either both the *galK* +85 and *galK* +275 mutations or the *galK* +85 mutation alone, which were re-plated on Luria Broth (LB)-agar plates and screened again to obtain an isogenic colony. This process was repeated again with oligo *galK* +85R (to remove the stop codon) and screened on galactose selective media, then finally with oligo *I44R* and screened on to re-introduce the amber mutation at the control mismatch site. Mutations were confirmed *via* Sanger sequencing.

Generation of HME61  $\Delta$ *uvrD* (a double MutH endonuclease and UvrD helicase knockout) was performed by Red-mediated recombination using a PCR-generated double-stranded DNA

fragment to replace the HME61 *uvrD* gene between positions –80 and +2600 with a kanamycin resistance cassette [26]. The DNA fragment with 60 nt homologous sequences flanking the *uvrD* gene were generated first by performing PCR reaction on a plasmid possessing a kanamycin resistance cassette (pMS1088 was a gift from Marcelo Sousa (Addgene plasmid # 80159)) using primers

5'-

CGGTTGGCATCTCTGACCTCGCTGATATAATCATCACGTTAAGGGATTTTGGTCATG  
AAC-3' and 5'-

GAGATTCAGTTTGCAGTCTGTTTATTTTCTAAATACATTCAAATATGTATCC  
GC-3, followed by a subsequent PCR reaction of that product with primers

5'-

CGTTTACTGCCGCATCTGGAAATTTCCCGGTTGGCATCTCTGACCTCGCTGATATAAT  
CA-3' and

5'-

CGGCATCGTTCGTCTGGATGCCTTCAAACCAGTAAAGAGATTCAGTTTGCAGTGGCA  
GTC-3'. After Red-mediated recombination, successful transformants were screened by growth on kanamycin resistant LB-agar plates according to the standard protocol.

### 2.3. “Semi-protected oligonucleotide recombination” (SPORE) assay

SPORE assays were performed as previously described [16]. Briefly, *E. coli* strains were made electrocompetent for Red-mediated recombination and stored at –80 °C until use. 50 µL of electrocompetent bacteria were thawed on ice and gently mixed with 2 µL of 100 µM in H<sub>2</sub>O of oligo 5'-*GT*, oligo 3'-*GT* (Table S1) to investigate 5'- coordinated or 3'- coordinated repair on the lagging strand, respectively, or, oligo (5)3'-*GT* (see Fig. 3) to investigate the relative rates of 5'- vs. 3'- coordinated LPR. The mixtures were electroporated at 1.8 kV using a GenePulser Xcell electroporation system, then immediately mixed with 1 mL room-temperature LB and grown for 30 min at 30 °C with shaking. After 30 min, the bacteria were spun down at 13500 × g for 15 s, the medium was decanted, then the bacteria washed in 1 mL of M9 minimal media and spun down again. After decanting, the bacteria were re-suspended in 1000 µL of M63 media, divided into two samples of 500 µL in 1.5 mL centrifuge tubes, and incubated at 30 °C with shaking for 72 h (although we note that in some cases HME61 samples were grown for 96 h in an attempt to enrich the fraction of transformed bacteria). The bacteria were then spun down at 13,500 × g for 3 min, and re-suspended in 20 µL 25% glycerol and stored at –80C. 2 µL of thawed bacterial stocks were then used directly to PCR a segment of the *galK* gene using Taq polymerase in 40 µL reactions using primers 5'-ACAATCTCTGTTTGCCAACG-3' and 5'-GGCTGGCTGCTGGAAG-3'. The reaction mixture was then sent for purification and Sanger sequencing by Eton Biosciences at its North Carolina branch (Durham, NC) using sequencing primer 5'-ACAATCTCTGTTTGCCAACG-3'.

### 2.4. Quantitative analysis of SPORE assays

#### 2.4.1. Maximum likelihood estimation of MMR activity from noisy sequencing chromatograms

A maximum likelihood estimation (MLE) was performed across all the samples performed for a given oligo on a specific strain by estimating the relative populations containing the wild-type sequence at the probe mismatch site *vs.* the probe mutation, in a manner that takes chromatogram noise, transformation efficiency, and non-specific oligo degradation into consideration (Figs. 1D, S1, and S2; see Supplementary Material for further discussion). In practice, this is an improved version of the analytical protocol first reported in Ref. [16], which was modified to allow us to robustly incorporate data from experiments with chromatograms containing higher levels of noise and/or experiments resulting in lower oligonucleotide transformation efficiencies. Furthermore, to compare the results of SPORE assays on strains first reported in this manuscript to those that previously were reported (for example, to strains SIMD50 and SIMD90, the wild-type and *mutS*-knockout strains, respectively), these previously-reported data were re-analyzed with this updated technique and additional SPORE trials performed on those strains have also been reported here for the first time. The re-analysis and inclusion of new SPORE data for those previously-reported strains were consistent with the previous report, albeit with improved confidence intervals.

To perform this analysis, briefly, we determine the fraction of the bacterial population that contains the wild-type *vs.* mutated sequence at the probe site mutation site from the sequencing chromatogram, and then determine the MMR repair efficiency by taking into account the oligo transformation efficiency, which is determined by the extracted fraction of the population with the control mutation at the control mismatch site, and the possibility of non-specific SPORE oligo degradation unrelated to MMR, which is determined by comparing the strain of interest to the *mutS*-knockout strains. This analysis also considers the level of noise in the sequencing chromatogram by considering the level of non-peak signals flanking the sites of interest. This information is then used to estimate a range of possible ‘true’ signal values at the probe and control mutation sites, such that the signals in lower-noise chromatograms are more likely to reflect the actual relative populations of bacteria with mutations at those sites. This process is shown graphically (Figs. S1–S4) and a pseudo-code of the computational protocol for data analysis is provided in the Supplementary Materials.

#### 2.4.2. Estimate of noise levels in sequencing channels and of oligonucleotide transformation efficiencies

Raw chromatogram data from the sequencing reads were imported into MATLAB (MathWorks; Natick, MA). The empirical cumulative distribution of the noise level for each sequencing channel is determined by the signal strengths at peak sites where that channel is not called. A probabilistic estimate for the ‘actual’ signal of the wild-type and control mutation channels at the control mismatch site (given an experimental chromatogram) is then generated by inverse transform sampling ( $1 \times 10^7$  trials) of these empirical cumulative distributions. The numerical results of the inverse transform samplings are binned by 0.001. From those distributions we then generate an empirical probability density function to estimate the true fraction of the population  $T$  with the control mutation, given the level of noise and relative signal strengths at the control mismatch site in the experimental chromatogram (Fig. S3). This analysis is performed similarly for the true wild-type sequence fraction at the probe site ( $S$ ) but using the probe mutation sites and probe mutation *vs.* wild-type sequence (Fig. S4).

#### 2.4.3. Estimate of the fraction of probes degraded non-specifically (degradation unrelated to MMR)

The fraction of the population with the wild-type sequence at the probe site mutation  $S$  is assumed to be:  $S = (1 - T) + T * D + T * R$ ; where  $T$  is the fraction of the population with the ‘control’ mutation (that was transformed by the SPORE oligo),  $D$  is the fraction of the transformed population where the probe mismatch was degraded nonspecifically [27] and  $R$  is the fraction of the transformed population where the probe mismatch was repaired by MMR. A likelihood function that describes the probability that a fraction of transformed oligos have been non-specifically degraded at the probe site  $D$  is found by performing this analysis on all the experiments performed using SIMD90 (inactive MMR,  $R = 0$ ). To clarify, the equation  $S = (1 - T) + T * D$  is numerically solved for  $D$  using a set of  $1 \times 10^7$  potential values of  $S$  and  $T$  generated from their respective empirical density functions determined for each experiment (determined as in the previous paragraph). The results are binned the results by 0.001, and the likelihood function of  $D$  is empirically generated by taking the bin-by-bin product across all of the SIMD90 experiments that were performed with the same SPORE oligo (5'-GT or 3'-GT).

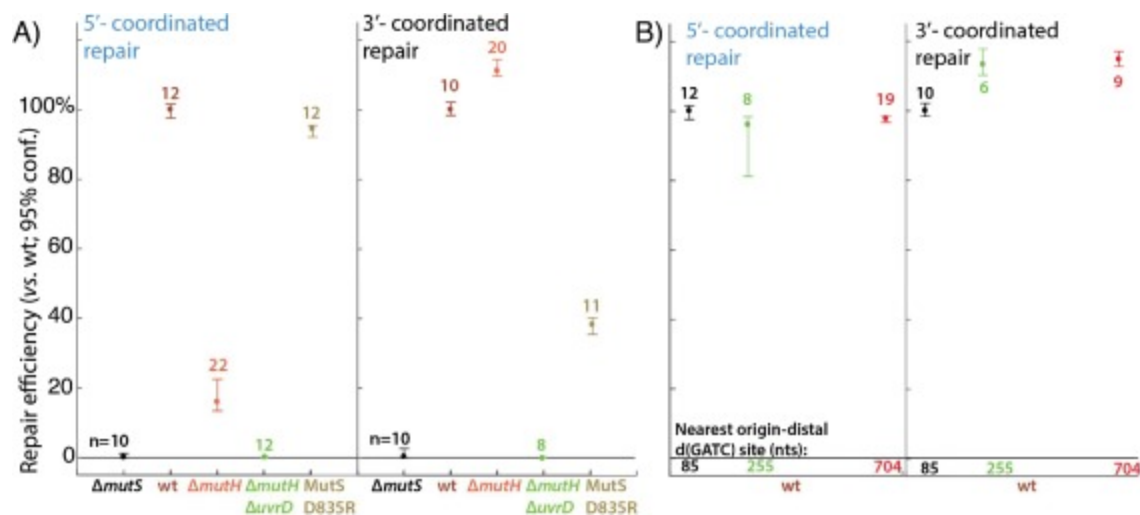
#### 2.4.4. Estimate of MMR repair efficiencies

For each of the strains, the likelihood function that describes the most likely population-level repair efficiencies given the experimental data  $R$  (Fig. S4) is then calculated similarly for each oligo and strain by instead solving for  $R$ , using the inverse transform sampling of the appropriate likelihood function of  $D$  to estimate what fraction of ‘false positives’ occur as a result of non-specific probe degradation. 95% confidence of the maximum likelihood estimate found by likelihood ratio test (Figs. S5–S10) (Chi-squared for 95% confidence; 1 degree of freedom). The analysis of repair of a 5'- ‘silent’ probe mutation (see Fig. 3) introduced on the opposite side of the control mismatch as the 3'- ‘probe’ mismatch on oligo (5)3-GT is performed similarly.

### 3. Results

#### 3.1. Repair of mismatches on the lagging strand can occur in the absence of MMR endonuclease MutH *in vivo*

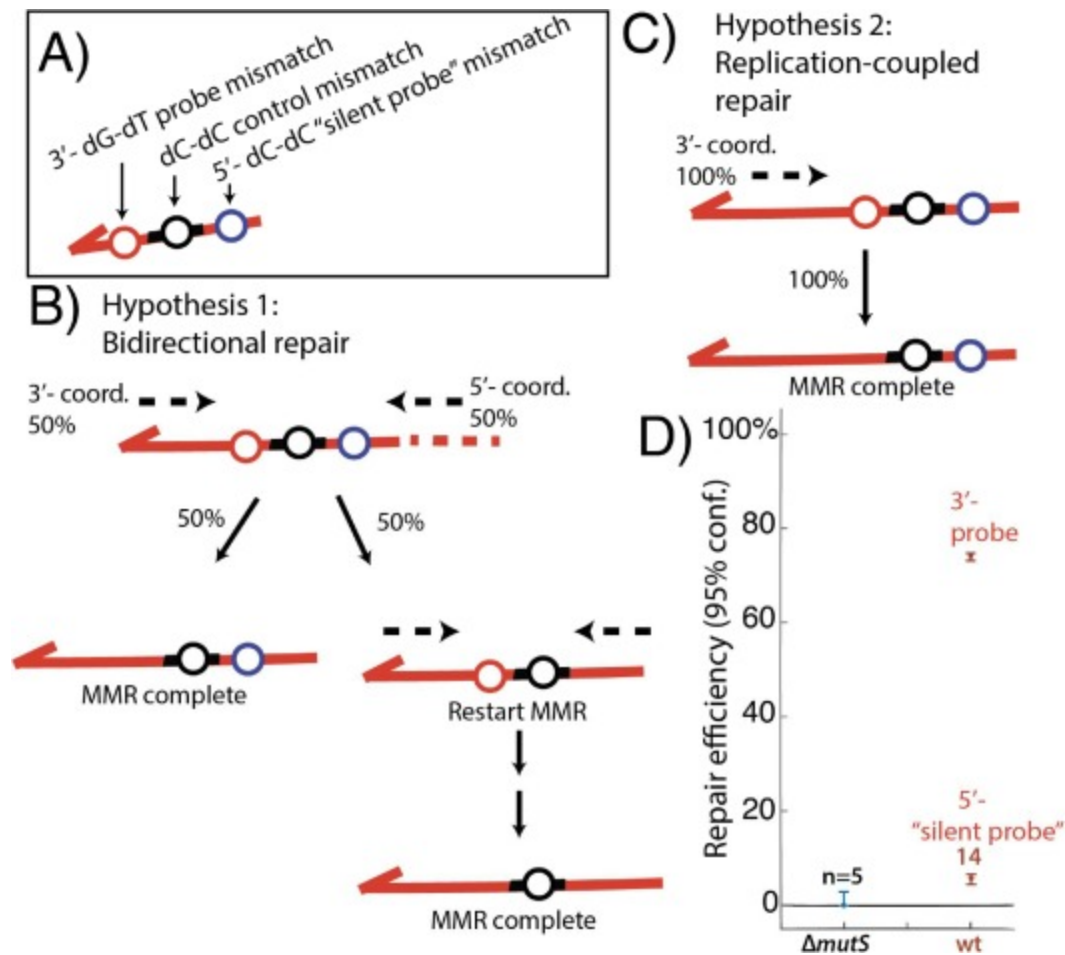
We performed our newly-developed SPORE assay [16] to directly and robustly quantify lagging-strand MMR efficiencies (Fig. 2). In a SPORE assay, living *E. coli* are transfected with synthetic 70-nucleotide-long oligonucleotides. SPORE oligos are complementary to the lagging strand template, except at sites designed to contain (i) a chemically-protected, MMR-refractory ‘control’ mismatch that confers a selective phenotype and (ii) an unprotected, MMR-active ‘probe’ mismatch, located 20 nt away either 5'- or 3'- of the control mismatch, such that the probe mismatch can only be repaired from that side (Fig. 1B). The ‘probe’ mismatch introduces a silent mutation. After transfection, these oligos can become incorporated into the *E. coli* genome during replication in a process that is proofread by the MMR pathway ([16] and references therein) (Fig. 1D).



**Figure 2.** A SPORE assay reveals efficient endonuclease-dependent and endonuclease-independent mismatch repair processes on the lagging strand. (A) Knockout of the gene for *E. coli* MMR nicking endonuclease MutH ( $\Delta mutH$ ) strongly decreases MMR coordinated from the 5'- end (left) while slightly increasing the efficiency of repair coordinated from 3'- strand discrimination signals (right), compared to wild-type (wt). (B) Endonuclease-dependent and endonuclease-independent repair processes on the lagging strand occur in a competitive manner. While endonuclease-dependent repair shows no dependence of MMR efficiency with changes in the distance to the nearest 5'- (origin-distal) d(GATC) site located up to 704 nucleotides (nts) away from the probe mismatch (left) [30], endonuclease-independent repair initiated from the origin-proximal end is increased in wild-type MMR strains as the origin-distal d(GATC) sites are removed (right). Dots are maximum likelihood estimate (MLE) of repair efficiency across all samples (number of samples  $n$  listed above each point). Error bars are 95% confidence derived from a likelihood ratio test, see Experimental Procedures and Supplementary Materials for details. Note that for  $\Delta mutH \Delta uvrD$  strains error bars are obscured by the image resolution.

Following growth in a selective media to enrich the population with the control mutation and Sanger sequencing of the transfected bacterial pools, we perform a quantitative analysis of the chromatograms that estimates the repair rate of the probe mismatches across the trial population in a manner that takes imperfect oligo transformation efficiency into account by simultaneously analyzing the mutation rate at the control mismatch site (see Methods and Supplementary Materials for details). The possibility of non-specific (unrelated to MMR) degradation of the SPORE oligo at the probe mismatch site is considered by comparison of the results of a SPORE assay using that same oligo in a *mutS*-knockout (MMR inactive) strain (Fig. 1D). Non-specific degradation at the probe site for 5'- mismatches was minimal (0.2% probe site mismatch degradation; 95% confidence 0%–1.5%;  $n = 10$ ) but expectedly [27] a little higher for oligos with a 3'- probe site (17.5% probe site mismatch degradation; 95% confidence 15.5%–18.3%;  $n = 10$ ). For a detailed description regarding how these effects are quantitatively considered during the analysis of the chromatograms and for a pseudo-code of the analysis protocol, see Materials and Methods and Supplementary Material. See also the Supplementary Data for results and analysis of all SPORE assay experiments.





**Figure 3.** A SPORE assay to differentiate the relative propensities of 5'- vs. 3'- coordinated LPR events. (A) In this assay, the SPORE oligo possesses a 3'- MMR-active (dG-dT) 'probe' mismatch; a chemically-protected, MMR-latent (dC-dC) 'control' mismatch that introduces a selectable mutation; and a 5'- MMR-latent (dC-dC) 'silent probe' that is not protected from 5'- coordinated LPR events. If MMR on the lagging-strand occurs bi-directionally (B), a significant fraction of the transformed population will lack the 'silent probe' mutation if it is excised/re-synthesized prior to the MMR-active probe mismatch being repaired. (C) Conversely, if MMR is coordinated unidirectionally in the direction of DNA replication, we would not expect any 'repair' of the 'silent probe' mismatches. (D) The results of the SPORE assay show that despite robust repair of the 3'- probe mismatch (74%), the 'silent probe' is only repaired ~5% of the time, suggesting MMR of lagging strand mismatches primarily occurs through 3'- LPR events.

To investigate the possibility of endonuclease-independent MMR, we performed a SPORE assay on a strain of *E. coli* where the gene for MMR endonuclease MutH had been removed from its genome (strain HME61) [25] (Fig. 2A). We found in this strain that repair of dG-dT mismatches coordinated from the 3'- end actually exhibited a slight increase in repair efficiency compared to wild-type (wt) strains (111.1% vs. wt; 95% confidence 109.7%–114%; n = 20). However, MMR coordinated from the 5'- end was significantly abrogated in the *mutH*-knockout strain, with 15.9% repair efficiency compared to wild-type MMR (95% confidence: 13.4%–22.4%; n = 22). These results would therefore put a lower bound for MutH-independent MMR activity on lagging-strand dG-dT mismatches at 76.9% repair efficiency (95% confidence: 75.9%–79.1%;

n = 20), compared to the 0.1% repair efficiency in the *mutS*-knockout strain (95% confidence: 0%–1.8%, n = 10; see Supplementary Data). While this finding directly demonstrates the existence of endonuclease-independent MMR on the lagging strand for the first time, our results would suggest that the 5'- ends of Okazaki fragments are not efficiently used to coordinate repair in a MMR endonuclease-independent manner, invalidating an earlier model of an endonuclease-independent MMR process we had proposed in a previous publication [16].

We will note that dG-dT mismatches can also be corrected in an MutS/MutL-dependent mechanism known as very-short patch (VSP) repair, which uses an alternative endonuclease known as VSR [28], [29]. However, VSP repair cannot explain the observed repair of dG-dT mismatches in the *mutH*-knockout strains because it always corrects the dT to a dC, which in the context of our SPORE assay would provide a false negative (through the successful introduction of a dG mutation) rather than the proper correction of a mismatched dG to a dA.

### 3.2. Endonuclease-dependent and endonuclease-independent MMR are coordinated asymmetrically along the lagging strand

We had previously observed directional asymmetries, similar to the MMR phenotype reported above for the *mutH*-knockout strain, in the ability to coordinate 5'- vs. 3'- repair in other MMR-mutant strains. This effect was previously seen to have the largest effect in a strain with a genomic mutation in the *mutS* gene (*mutS D835R*) that disrupts the stoichiometry of MutS complexes [31], [32]. MutS associates as a homodimer or a homotetramer (dimer of dimers) in solution, with the homotetrameric form being the predominant species [33]. However, tetrameric MutS was not believed to be essential for MMR, because strains with a genomic *mutS D835R* mutation—which disrupts the ability of MutS to tetramerize but not its ability to dimerize—was found only to result in a moderate increase in the rate of spontaneous mutation *vs.* *mutS* knockout [31]. Unexpectedly, using the SPORE assay we had previously found that the strains with the *mutS D835R* mutation did indeed suffer a significant repair defect *in vivo* in 3'-coordinated repair, but not 5'- coordinated MMR. This asymmetry is now suggestive of a possible relationship to MutH-independent repair. In order to directly compare those previous results with the *mutH*- knockout strain, here we reanalyzed the previously-reported results of SPORE assays using the *mutS D835R* strain and included additional experimental trials (Fig. 2A; Supplementary Data).

As before, in the *mutS D835R* strain, repair from 5'- was minimally affected (94.5% repair efficiency *vs.* wt; 95% confidence: 92.2%–95.5%; n = 12), while repair efficiency from 3'- dropped by two-thirds (38.2% repair efficiency *vs.* wt; 95% confidence: 35.5%–40.2%; n = 11) (Fig. 2A). Taken together with the findings of the previous section, these results would suggest that there are differentiated mechanisms of MMR on the lagging strand: an endonuclease-dependent MMR which utilizes dimeric MutS, and an endonuclease-independent MMR—coordinated primarily from the 3'- orientation, or the direction of lagging-strand replication—which employs a tetrameric form of MutS (*i.e.*, see Fig. 5).

### 3.3. Endonuclease-dependent and endonuclease-independent repair processes are competitive or mutually exclusive

The increase in 3'-coordinated repair in the *mutH*-knockout strain HME61 suggests that endonuclease-independent and endonuclease-dependent repair may be competitive processes on the lagging strand. In the canonical *E. coli* MMR pathway [2], MutH introduces single-strand breaks (SSBs) into DNA at the nearest hemi-methylated d(GATC) site on the strand that is transiently unmethylated immediately after replication [30]. For this study we generated a series of strains where the nearby origin-distal d(GATC) sites—5'- of the mismatch (Fig. 1C), in the endonuclease-dependent direction of repair—were altered *via* silent mutations to determine the effects of removing the nearby substrates for MutH on both endonuclease-dependent and endonuclease-independent (3'-coordinated) MMR.

Consistent with previous studies, which showed no effect on repair efficiencies when origin-distal mismatches were less than ~1000 nt away from the repair site [30], we see no significant decrease in MMR efficiency with the nearest origin-distal d(GATC) site located up to 704 nt away from the inserted mismatch (Fig. 2B). However, increasing the distance to the nearest 5'-d(GATC) site does have the anomalous effect of increasing the 3'-coordinated repair in wild-type strains to levels of *mutH*-knockout strains. The increase in (endonuclease-independent) 3'-coordinated repair as the nearest substrates for MutH nicking are removed show that these repair processes are competitive, or at least mutually exclusive, on the lagging strand.

#### 3.4. Repair coordinated from 3'- of the mismatch is the primary mechanism of lagging-strand MMR

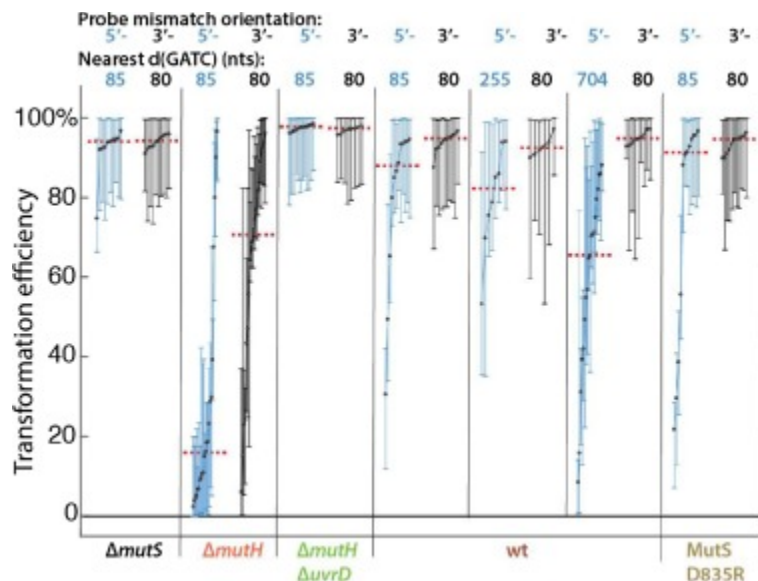
While the SPORE assays as described above can resolve the overall repair efficiencies of 5'- or 3'-coordinated LPR events independently, to determine their relative propensities on the lagging strand we performed a SPORE assay using an alternative SPORE oligo. Oligo (5)3-GT (Fig. 3A) introduces three mismatches when bound to the lagging-strand template: (i) a 3'-dG-dT 'probe' mismatch and (ii) chemically-protected MMR-latent dC-dC 'control' mismatch, as before, as well as (iii) an unprotected 5'-dC-dC mismatch which also introduces a silent mutation into the *galK* gene. While the 5'- 'silent probe' mismatch is not expected to excite MMR, by virtue of the silent mutations it can introduce it can serve as a probe to differentiate two models of repair: if repair occurs bi-directionally (Fig. 3B), as has long been suggested based on biochemical studies [3], [34], then 5'-coordinated LPR would be just as likely to initiate as 3'-coordinated LPR, removing the 'silent probe' prior to MMR re-initiating in a significant fraction of the population before successfully removing the MMR-activating 3'-probe mismatch. Alternatively, if MMR on the lagging strand is coordinated by the directional motion of the DNA polymerase during replication [30] (Fig. 3C), we would not expect a 5'- 'silent probe' to ever be excised/re-synthesized before the aggravating 3'-probe mismatch.

Using oligo (5)3-GT, we found that the 3'-probe mismatch was repaired 74% overall (95% confidence: 73.0%–74.6%; n = 14)—consistent with oligo 3'-GT—but repair of the 'silent probe' occurred only 5.8% of the time (95% confidence: 4.5%–7.2%, n = 14) relative to *mutS*-knockout control (0.1%; 95% confidence: 0.0%–2.9%; n = 5). This result would suggest that >90% (between ~92% and 93%) of LPR events are initially coordinated from the 3'-side of a lagging-strand mismatch.

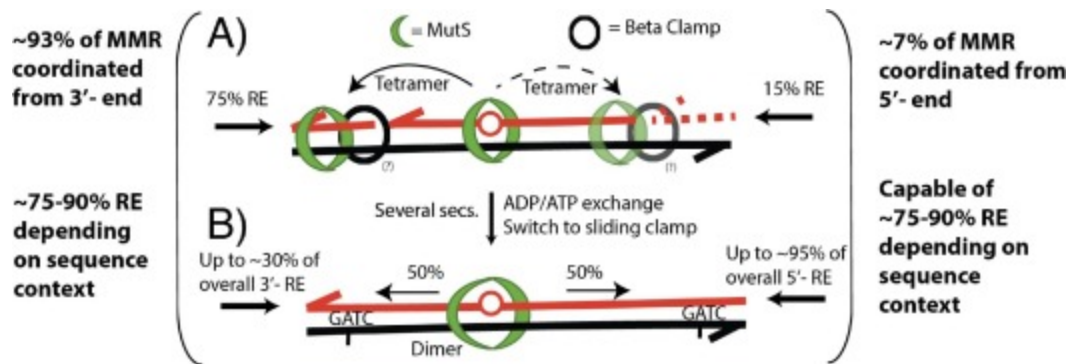
Considered in light of our finding that 3'-coordinated MMR is highly efficient in the absence of MutH, these results would suggest that 3'-coordinated, MutH-independent LPR is the primary mechanism of lagging-strand MMR.

### 3.5. Endonuclease-dependent repair processes modulate genomic stability during lagging-strand MMR

During the course of these experiments, we noticed that in strains with fully-functional (wild-type, wt) MMR the fraction of the populations with the control mutation after enrichment in galactose media—estimated by the ratio of control mutation signals to wild-type signal (see Supplementary Material)—dropped when repair was blocked from the 3'-orientation using oligo 5'-GT (Fig. 4). This effect appeared significant, relative to the uniformly high, near-total fractions of the enriched population with the control mutation observed in experiments with oligo 3'-GT that blocked repair from the 5'-end. *mutS*-knockout strain SIMD90 exhibited this high apparent 'transformation efficiency' regardless of oligo used, while *mutH*-knockout strain HME61 exhibited poor apparent transformation efficiencies when repair was blocked from either direction (but particularly for oligo 5'-GT), to the extent that some SPORE assays using HME61 often required extended enrichment to increase the relative fraction of the population with the control mismatch so that a more robust analysis of repair events could be performed.



**Figure 4.** Transformation efficiencies for different strains and repair processes. In strains with wild-type (wt) MMR, transformation efficiencies were asymmetric and consistently poorer when 3'-repair was blocked, but not when 5'-repair is blocked. *mutH*-knockout strains exhibit consistently poor transformation efficiencies, except in strains where the gene for helicase UvrD is also removed (green), which suggests the SPORE oligonucleotides are not removed *via* strand-displacement synthesis. Note that some SPORE assays using *mutH*-knockout strains involved an extended enrichment so that a more robust analysis of repair events could be performed. Points are median estimated transformation efficiency for each SPORE chromatogram (e.g. Fig. S3) and dotted lines are median across all samples. Error bars are 95% confidence of transformation efficiency estimates based on chromatogram analysis.



**Figure 5.** A two-stage model for the repair of dG-dT mismatches on the lagging strand suggested by the results of the SPORE assay: an endonuclease-independent MMR which employs a tetrameric form of MutS is the primary mechanism of lagging strand MMR, perhaps initiated by a mismatch-bound MutS dimer able to tetramerize with a MutS dimer released at the 3'- end of a recently replicated Okazaki fragment [47] (MutS at the 5'- end of the Okazaki fragment may appear later after the next fragment is synthesized). If replication-coupled MMR fails, a mismatch-bound MutS dimer may convert to a sliding clamp and detach from the mismatch by rapidly and randomly diffusing along the DNA molecule with MutL and MutH [46] to identify a hemi-methylated d(GATC) site (either 5'- or 3'- of the mismatch) and initiate repair. RE = repair efficiency.

Since we were enriching for successful transformants by enrichment of *galK*<sup>+</sup> mutants vs. *galK*<sup>-</sup> background population, these poor transformation efficiencies would suggest that under some conditions the control mutation becomes more likely to be lost in an MMR-dependent manner. This loss could be caused by either a direct removal of the entire SPORE oligonucleotide prior to its incorporation into the genome; or because of decreased genomic stability during MMR that could result in double-strand breaks/genomic recombination events (which could cause a loss of the control mutation) or in the death of transformants. Because the transformation efficiencies of oligo 5'-GT dropped sharply and monotonically as the distance to the nearest 5'- d(GATC) site increased from 85 bp to 704 bp (Fig. 4), this trend would suggest that the reduction of transformation efficiency is at least partly related to the long-patch repair (LPR) mechanism, since there are no d(GATC) sites present in the SPORE oligos. Because the results presented in Fig. 3 suggest that 3'- coordinated LPR is attempted first when MMR on the lagging strand is excited (Fig. 3), it is likely that by blocking the 3'- coordinated repair mechanism with the 5'-GT oligo we then force the MMR process to restart in way that allows 5'-coordinated repair, which strongly depends on endonuclease activity to occur. Hence, endonuclease-dependent MMR where a large 'long-patch' excision tract is required for repair—or where the probability of even identifying a hemi-methylated d(GATC) site before it is fully methylated is lower, rendering a detected mismatch 'unrepairable' [35]—may introduce genomic stress, decrease genomic stability, and promote the loss of the control mutation.

Alternatively, because the MMR protein MutL—the 'linker' protein in *E. coli* which stimulates MutH nicking activity after MutS identifies a mismatch [6], [9]—also interacts with and stimulates the activity of MMR helicase UvrD [36], in principle it is possible that MutS/MutL-stimulated UvrD helicase activity could become more prevalent in the absence of MutH. This could occur before LPR was even attempted, and if knockout of MutH increases the probability that the SPORE oligo will be removed by this mechanism [37] before its integration into the

genome, this process would also reduce the apparent transformation efficiency. At this point we cannot directly resolve how often these rapid events may be occurring in wt strains or how much it may be stimulated by the absence of MutH.

Another possibility is that the SPORE oligo and its control mismatch would be removed by a strand displacement synthesis-like mechanism [13], [38], where a DNA polymerase is recruited to re-synthesize the erroneous lagging strand, the force of which displaces the previous strand. However, this does not appear likely, as knockout of the gene for the UvrD helicase in *mutH*-knockout strains restored transformation efficiencies back to high levels (Fig. 4), eliminating the possibility that the oligo is directly removed by this displacement mechanism during repair. *uvrD* knockout also resulted in the total elimination of lagging-strand MMR in *mutH*-knockout strains (Fig. 2A), which would confirm that MutH-independent repair likely follows the canonical MMR excision mechanism of unwinding by UvrD followed by exonucleolytic error removal.

Overall, the observed decreases of transformation efficiency under certain conditions are likely a result of (i) decreased genomic stability during MMR when endonuclease-dependent repair is challenged, delayed, or eliminated, resulting in a loss of the control mutation, (ii) a potential increase in MutS/MutL-stimulated removal of the SPORE oligo by UvrD, and/or (iii) a combination of the two factors.

#### 4. Discussion

While there have been some reports in *E. coli* suggestive of an endonuclease-independent mismatch repair mechanism on the lagging strand—[39], [53], using the SPORE assay we have presented direct evidence for the first time of a mismatch repair process on the lagging strand that is independent of MMR endonuclease MutH. As noted above, based on the results of the SPORE assay there appear to be two differentiated mechanisms of lagging-strand MMR: (i) an endonuclease-independent MMR that employs a tetrameric form of MutS, is coordinated primarily from 3'- of a mismatch, and is likely the primary mechanism of lagging strand repair; and (ii) a MutH-dependent MMR mechanism that utilizes a dimeric form of MutS.

Because DNA polymerases replicate DNA by adding nucleotides in the 3'- direction, the 3'- bias in endonuclease-independent MMR activities might also suggest that the MutH-independent MMR process is coordinated with the replisome as replication is occurring, similarly to what has been proposed to occur in d(GATC)-dependent repair *in vivo* [30]. We do note that this asymmetry during repair is in stark contrast to what is observed for *in vitro* MMR reactions performed on non-replicating DNA, which are found to be effectively bidirectional [3], [34], [40], [41], or equally efficient in repairing mismatches in DNA with 5'- or 3'- d(GATC) sites or SSBs. Furthermore, while additional work is required to directly identify the specific epigenetic signals used during the endonuclease-independent MMR process to discriminate the newly-replicated strand from the template strand, the most likely candidates for these signals are the single-strand breaks between Okazaki fragments before they have been ligated together [13].

An additional curious feature identified from SPORE assay data is that while ~95% of lagging-strand MMR activity originally initiates from 3'- long patch repair (LPR) events, 5'- mismatches on SPORE oligos are also repaired with high efficiency (~80–90% repair) even when 3'- LPR is

blocked. This would suggest that while 3'-LPR is attempted first, there exists an efficient “back-up” MMR mechanism that is also capable of coordinating repair from 5'- of the mismatch. Such ‘two-stage’ behavior is also known to be performed by MutS *in vitro*: it has been observed on non-replicating DNA that after a MutS dimer binds to a mismatch, several seconds later the MutS dimer undergoes an ATP/ADP exchange and a structural change to a ‘sliding clamp’ [7], [42], [43], [44]. The MutS sliding clamp then releases from the mismatch to rapidly, randomly, and bidirectionally diffuse along the DNA molecule [44], [45], and *in vitro* these MutS sliding clamps were recently observed using single-molecule fluorescence experiments interacting with complexes of MutL and MutH as they diffused together along a DNA molecule containing a mismatch [46]. Based on the SPORE data, it may be that if the main, replication-coupled MMR process that is coordinated from 3'- of a mismatch fails, a second ‘bidirectional’ attempt at repair is made. Since, by that time the MutS sliding clamp releases from a mismatch the nearby section of the lagging strand may have already been ligated into a continuous strand, this back-up mechanism would then require endonuclease MutH to introduce a *de novo* SSB at hemi-methylated d(GATC) sites in order to initiate repair (Fig. 5). Fig. 5 summarizes the relative repair efficiencies (RE), propensities, and polarities of MutH-dependent and -independent repairs suggested by the results of the SPORE assay, which overall are suggestive of this ‘two-stage’ model.

As can be seen from this study, the SPORE assay provides a direct way to introduce targeted mismatches directly into the replicating genome and to decouple repair efficiency from transformation efficiency. As a result, this assay is capable of revealing new details that can be obscured by traditional assays for MMR *in vivo* such as spontaneous reversion assays (which provide estimates of overall cellular mutational rates), and allows new ways to deconstructing MMR mechanisms which may be redundant or have multiple (back-up) mechanisms *in vivo* (Fig. 5). We will note that, in addition to new SPORE oligo designs and optimizations, there are further opportunities to expand and improve on the assay. For example, while the dC-dC mismatches introduced as the ‘control’ mismatch by the SPORE oligo are very weakly recognized by MutS and their presence do not prompt the initiation of mismatch repair [43], [48], we cannot rule out the possibility that the weak interaction between MutS and dC-dC may influence the MMR reaction during a SPORE assay; the recent identification by van Ravesteyn et al. [49] that mismatches introduced using locked nucleic acids (LNAs) are not recognized by *E. coli* MutS (binding as strongly to those as to homoduplex DNA) may indicate that chemically-modified oligonucleotides could represent a better choice for the ‘control’ mismatch. Additionally, because the oligonucleotide recombination techniques on which SPORE is based are commonly performed in yeast [50], [51] and mammalian cells [49], [52], we expect this assay will be useful in elucidating replication-coupled aspects of eukaryotic mismatch repair processes *in vivo* as well.

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## **Appendix A.** Supplementary data

Supplementary data associated with this article can be found at  
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